Supplemental Material

Para- and Ortho-Substitutions are Key Determinants of Polybrominated Diphenyl Ether Activity Toward Ryanodine Receptors and Neurotoxicity

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Table of Contents

Methods 1

References 6

Figures and Legends 7

Methods

[^{3}H]Ryanodine binding assays. RyR1 activity was assessed using 25 μg/mL microsomal protein incubated in buffer containing 140mM KCl, 15mM NaCl, 20mM HEPES, 0.05mM $CaCl_2$, and 1nM [^{3}H]Ry, pH 7.4 (final volume = 500μL), unless otherwise noted. Nonspecific binding was assessed by addition of 1000-fold unlabeled ryanodine. [^{3}H]Ry binding experiments were performed by incubating to equilibrium at 37^{o}C for 3 hrs with constant shaking. RyR2 activity was assessed using 200μg/mL heart whole membrane preparation incubated in buffer with the same conditions as above except 5nM [^{3}H]Ry and 7μM $CaCl_2$ were used and the reaction mixture was incubated for 16 hr at 25^{o}C. Reactions were quenched by filtration as previously described (Pessah et al. 2006). Experiments were performed in triplicate using three different membrane preparations. Concentration-effect curves were fitted using the software Origin 7.0 (OriginLab Corporation, Northampton, MA) to calculate maximal occupancy. Statistical significance was determined using two-way ANOVA.

Microsomal Ca^{2+} flux measurements. Net Ca^{2+} efflux from actively loaded microsomal membrane vesicles was monitored by the metallochromic dye antipyrylazo-III (AP- III, at 250μM in 18.5mM MOPS, 92.5mM KCl, 7.5mM Na-pyrophosphate, pH 7.0). Microsomal vesicles isolated from skeletal muscle (50μg/mL) were actively loaded by serial additions of 20μM $CaCl_2$ in the presence of 1mM ATP, 5mM phosphocreatine and 10μg/mL creatine phosphokinase (active Ca^{2+} loading phase). Once the APIII dye signal returned to baseline (~100nM extravesicular Ca^{2+}), PBDE congeners or caffeine were introduced into the solution containing the Ca^{2+} loaded vesicles to assess their ability to trigger net Ca^{2+} release. The ability of rapamycin to inhibit BDE-4 induced Ca^{2+} release was determined by addition of 0 to 50μM rapamycin 2 min prior to active Ca^{2+} loading. FKBP12-independent RyR1 activity was assessed by addition of 10 mM caffeine (Wong and Pessah 1997). Pharmacological blockade of RyR1 was achieved by the addition of 1μM ruthenium red (RR) prior to introducing the PBDE. Absorbance signals were calibrated at the end of each experiment by addition of 1μg of the Ca^{2+} ionophore A23187 followed by 20μM additions of Ca^{2+} from a National Bureau of Standards stock. Experiments were performed in triplicate using three different membrane preparations. EC_{50} values and linear curve fitting to determine initial rates of Ca^{2+} release were obtained using the software Origin 7.0 (OriginLab Corporation, Northampton, MA). Statistical significance was determined using two-way ANOVA ($\alpha = 0.05$).

Ca^{2+} imaging of HEK293 cells. Human embryonic kidney (HEK293) cells null for RyR protein ($HEK293^{null}$) were maintained in DMEM medium supplemented with 2mM glutamine, 100μg/mL streptomycin, 100U/mL penicillin, 1mM sodium pyruvate, and 10% fetal bovine serum at 37ºC under 5% CO_2. Cells were transfected with the full-length cDNA sequence of either *ryr1* or *ryr2* cloned into pCI-neo expression vector (Promega, Madison, WI) using

Lipofectmanine 2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Stably transfected cells were obtained by Geneticin sulfate (G418, 800 μg/mL) selection for 2 weeks. Single colonies resistant to G418 were then transferred into a 24 well plate and tested for RyR1 expression using immunofluorescence staining with monoclonal antibody 34C (Sigma, St. Louis, MO) or C3-33 (a generous gift from Dr. Gerhard Meissner, University of North Carolina) that recognize RyR1 and RyR2, respectively. Clones stably expressing RyR1 ($HEK293^{RyR1}$) or RyR2 ($HEK293^{RyR2}$) were maintained in DMEM medium supplemented as described above with addition of 200µg/mL G418. Cells of each genotype were pre-treated with 250nM of BDE-47 or BDE-49 for 16 hr, then loaded with 5µM Fluo-4/AM (Invitrogen, Carlsbad, CA) at 37°C for 30 min in an imaging buffer consisting of 140mM NaCl, 5 mM KCl, 2mM $MgCl_2$, 2mM $CaCl_2$, 10mM HEPES and 10mM glucose, pH 7.4, supplemented with 0.05% bovine serum albumin. Cells were rinsed three times in imaging buffer, incubated for an additional 10 min at room temperature on an inverted stage IX-71 Olympus microscope (Center Valley, PA). Cytoplasmic Ca^{2+} transients ($[Ca^{2+}]_{cyt}$) were imaged with a cooled CCD camera (model 512B; Photometrics, Tucson, AZ) using a 40X objective. Image sequences were captured at 5Hz using EasyRatioPro software (Photon Technologies International, Birmingham, NJ). After obtaining baseline measurements, the response to a 10 sec focal application of caffeine or 4-chloro-m-cresol (4-CmC) was applied by a multichannel microperfusion system (AutoMate Scientific, Berkeley, CA). BDE-47 and BDE-49 were maintained in the imaging buffer during dye-loading and throughout acquisition of $[Ca^{2+}]_{cyt}$ data. The amplitude of $[Ca^{2+}]_{cyt}$ transients was calculated as $(F_{max}-F_{min})/F_{min}$. One-way ANOVA and post-hoc (Bonferroni's Multiple Comparison Test) analysis were performed using Graph Pad Prism Software (version 5.0)

Primary culture of mouse and rat cortical neurons. All animal procedures were performed under an approved UC Davis IACUC protocol. High density cultures of cortical neurons were dissociated from P0~P2 postnatal C57BL/6J (B6) mice. Cortices were incubated with trypsin (0.25%) at 37˚C for 20 min and washed three times with Hanks buffered saline (Ca^{2+}/Mg^{2+} free; HBSS) before dissociating the neurons with a fire-polished Pasteur pipette. The cell suspension was filtered through a 40μm Nylon-meshed cell strainer and cells in the filtrate were spun down at 200×g for 3~5 min and re-suspended with a fire-polished Pasteur pipette in Neurobasal medium (Invitrogen, Carlsbad, CA, USA) containing NS21 supplement (Chen et al. 2008), 0.5mM glutamine and 5% fetal bovine serum (FBS). Cells were plated at a density of 30,000 cells per well in a 96 well plate pre-coated with poly-L-lysine (0.01%, Sigma-Aldrich Chemical (St. Louis, MO) After 4 hr, the medium in each well was replaced with FBS-free Neurobasal media containing NS21 supplement and 0.5mM glutamine. Cells were maintained at 37˚C in a humidified environment of ambient air/5% CO_2. Half of the medium was changed after 4 days *in vitro* (4 DIV). PBDEs were added to the culture medium at 6 DIV and neuronal morphology and viability examined at 8 DIV (48 hr PBDE exposure).

Rat cortical neurons were dissociated from the neocortex of postnatal day 1 Sprague Dawley rat pups (Charles River Laboratories, Hollister, CA) as previously described (Yang et al. 2009). Dissociated cells were plated onto microelectrode arrays (MEA) with a central 0.88 mm^2 recording matrix of 64 microelectrodes (catalog #Med-P545A, AutoMate Scientific Inc, Berkeley, USA) that had been precoated with poly-L-lysine (0.5 mg/ml, Sigma Chemical Co., St. Louis, MO) and laminin (10 μg/mL, Invitrogen, Carlsbad, CA) at a density of 1×10^4 cells/MEA. Cultures were maintained in Neurobasal-A (Invitrogen) supplemented with B27 (Invitrogen) as

previously described (Wayman et al. 2006). Half of the medium was replaced with fresh Neurobasal-A with B27 twice weekly.

MEA recording and data analysis. After 3 weeks in culture, cortical neurons grown on MEAs were placed into the recording chambers of the MED 64CH Integrated Amplifier system (AutoMate Scientific Inc), which was connected to a Dell workstation loaded with Mobius software (Mobius 0.3.7 , AutoMate Scientific Inc, Berkeley, CA). Baseline activity was recorded for 10 minutes at 37°C. Cultures were then exposed to vehicle by adding 1 μL of undiluted DMSO into MEA cultures containing 1 mL of culture medium for a final concentration of 0.1% DMSO. After gently rocking the cultures to disperse the DMSO, activity was recorded for an additional 10 minutes at 37°C. Subsequently, cultures were exposed to either BDE-47 or BDE-49 by adding 1 μL of 1000X stock solution in DMSO to the well. Cultures were sequentially exposed to increasing concentrations of BDE, with activity recorded for 10 min following each addition. Raster plots of spike activity were analyzed using the Spike Sorting and DC remove filter applications in the Mobius software to identify and sort spikes. Spikes of activity >3 times the baseline level during recording were scored as spontaneous activity. The total number of spikes per electrode per 10 min recording session was determined and then averaged over the array (only active electrodes were included) to determine the mean spike number per MEA. Three MEAs from three independent dissections were analyzed per BDE.

Tetrazolium-based MTS assay. The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay was performed to test cell viability in cultured mouse cortical neurons using the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega Inc., Madison, WI). Briefly, dissociated mouse cortical neurons were plated in a 96 well plate at a density of 3×10^4 cells per well. After a 48 hr exposure to

BDE-47, BDE-49, or equivalent volume of vehicle (0.01% DMSO final), an endpoint measurement of viability was performed following the manufacturer's instructions, using a microplate reader (Molecular devices, Sunnyvale, CA) set at 490nm. Media plus CellTiter 96 ® AQueous One Solution reagents added to wells without cells were also measured as a negative control. Experiments were performed on three independent cultures, each vehicle or PBDE concentration replicated in 4 wells. Differences from vehicle were tested using two-way ANOVA with post-hoc analysis.

References

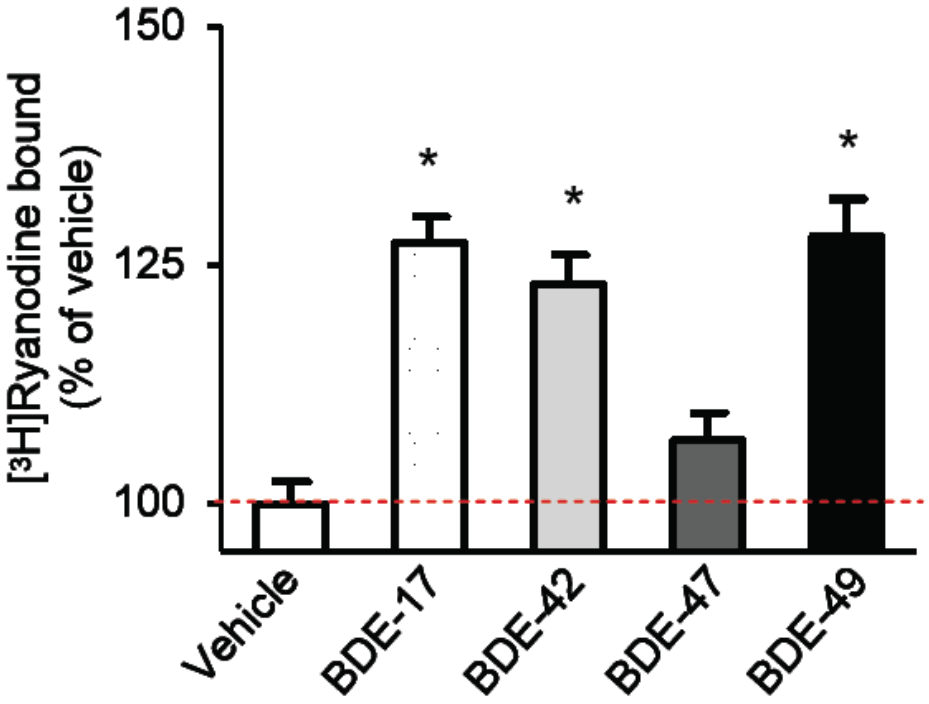
Chen Y, Stevens B, Chang J, Milbrandt J, Barres BA, Hell JW. 2008. Ns21: Re-defined and modified supplement b27 for neuronal cultures. J Neurosci Methods 171(2): 239-247.

Pessah IN, Hansen LG, Albertson TE, Garner CE, Ta TA, Do Z, et al. 2006. Structure-activity relationship for noncoplanar polychlorinated biphenyl congeners toward the ryanodine receptor-Ca^{2+} channel complex type 1 (ryr1). Chem Res Toxicol 19(1): 92-101.

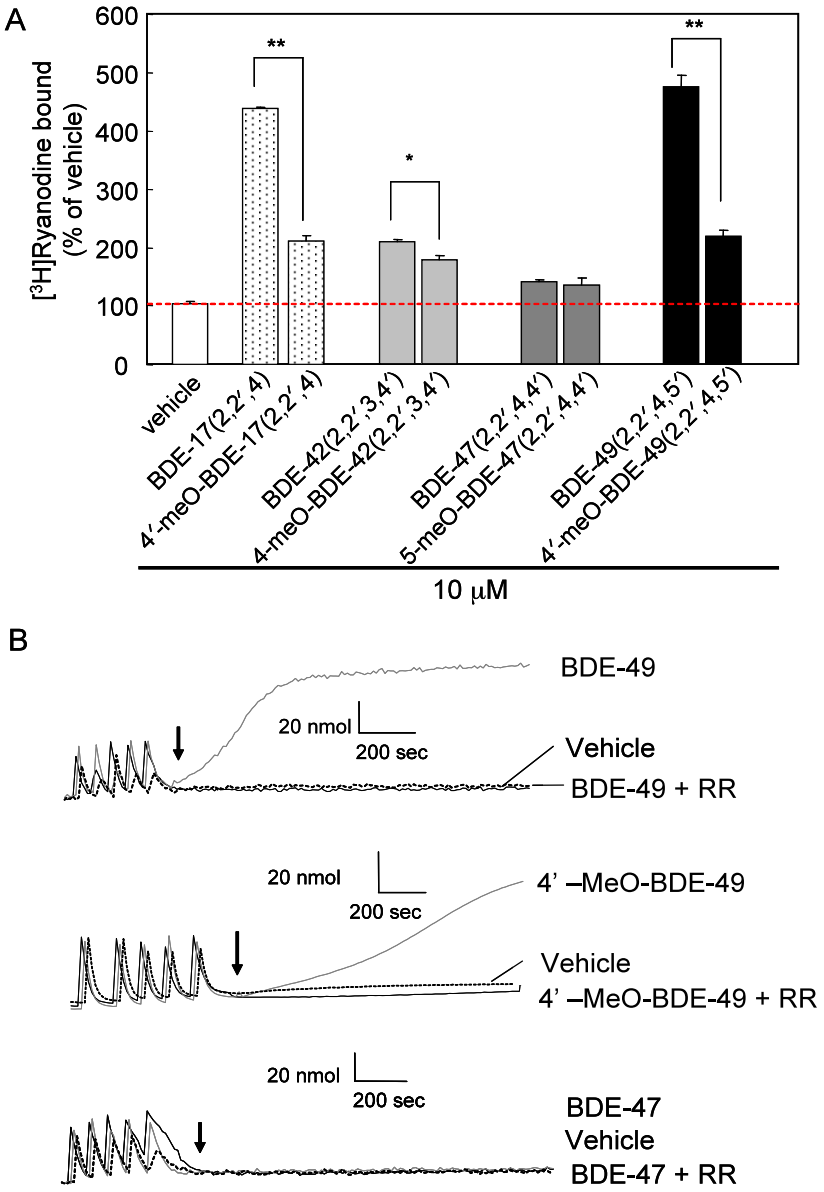
Wong PW, Pessah IN. 1997. Noncoplanar pcb 95 alters microsomal calcium transport by an immunophilin fkbp12-dependent mechanism. Mol Pharmacol 51(5): 693-702.

Yang D, Kim KH, Phimister A, Bachstetter AD, Ward TR, Stackman RW, et al. 2009. Developmental exposure to polychlorinated biphenyls interferes with experience-dependent dendritic plasticity and ryanodine receptor expression in weanling rats. Environ Health Perspect 117(3): 426-435.

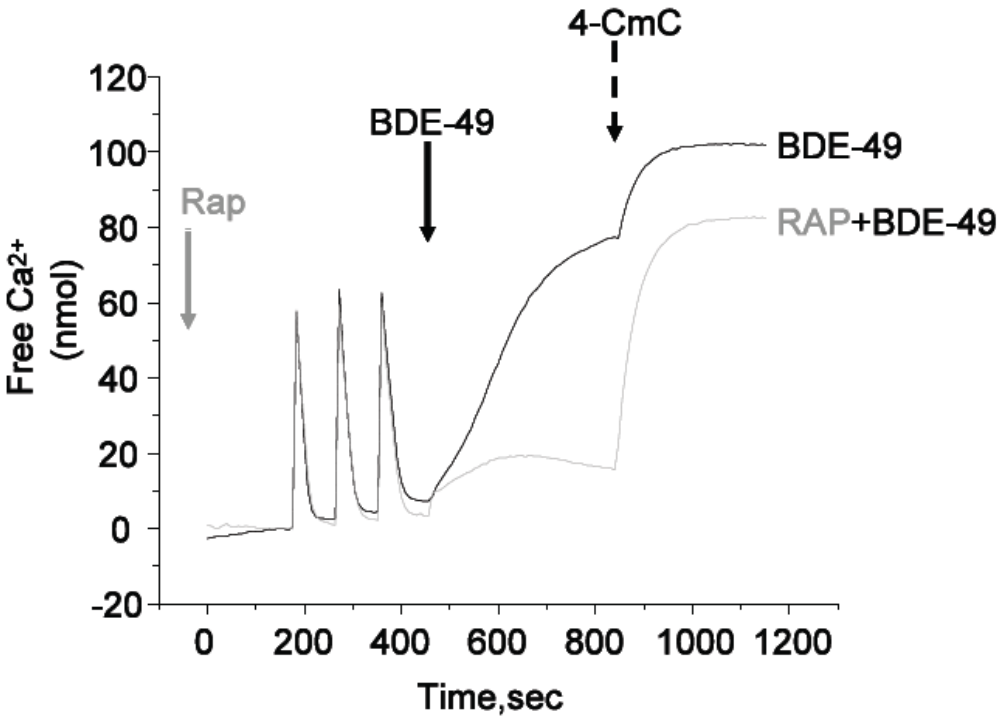
Supplemental Figures



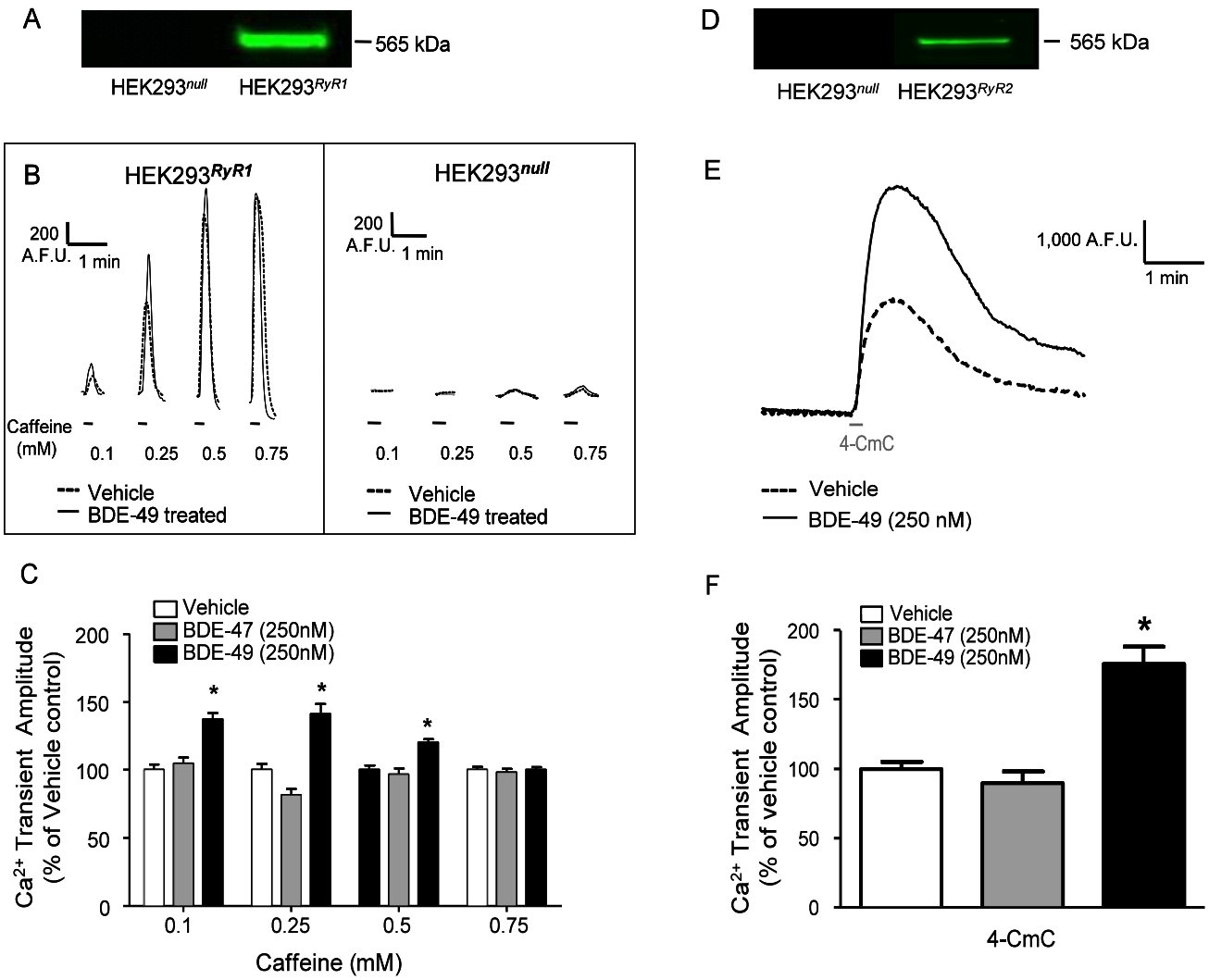
Supplemental Material, Figure 1; Structure-activity of PBDEs towards RyR activity in cortical membrane preparations. [^{3}H]Ry binding to cortical membranes in the absence and presence of 10 µM of BDE-17, -42, -47 or 49. Binding was performed with 100 µg/ml membranes prepared as previously described (Yang et al. 2009) in binding buffer containing 250mM KCl, 15mM NaCl, 20mM HEPES, 0.007mM CaCl2, 300mM sucrose and 5nM [3H]Ry, pH 7.4 (final volume = 500µL). Data are presented as mean ± S.E.M. (n=5 wells per culture day, N=2 independent culture days), *p<0.05



Supplemental Material, Figure 2; Activity of methoxy-BDEs towards RyR1. A Addition of one methoxy group at *para*-position of BDE-17, BDE-42, and BDE-49 significantly decreased the amount of [^{3}H]Ry binding compared to the corresponding parent. *$p<0.05$, **$p<0.01$. N=3. B After sequential loading of Ca^{2+}, introduction of 10 μM of BDE-49 or methoxy-BDE-49 triggered Ca^{2+} efflux from microsomal vesicles, whereas BDE-47 did not. Inhibition of RyR1 channels with 1μM ruthenium red (RR) prevented BDE-induced Ca^{2+} efflux. N=4



Supplemental Material, Figure 3; Rapamycin selectively interferes with BDE-49 induced Ca^{2+} release. Pretreatment of microsomal vesicles with rapamycin 2 min prior to loading vesicle with Ca^{2+} (red arrow) inhibited Ca^{2+} release triggered by subsequent addition (black arrow) of 2μM BDE-4, but did not inhibit 4-CmC-induced Ca^{2+} release. Gray arrow indicates the addition of 1 mM of 4-CmC. N=3.



Supplemental Material, Figure 4; BDE-49 enhances RyR agonist-triggered activation of HEK293^{RyR1} and HEK293^{RyR2} cells. A Western blotting using monoclonal antibody 34C recognizes RyR1 expression of RyR1 protein in HEK293 cells stably transfected with RyR1 (HEK293^{RyR1}), whereas HEK293^{null} did not show detectable RyR1 protein. B Sample traces of Fluo-4 fluorescent emission from individual cells pretreated with vehicle or BDE-49 (250nM) for 16 hr showing Ca^{2+} transients triggered by 10 sec focal application of caffeine (black bars). HEK293^{null} cells did not respond to caffeine with or without BDE pretreatment. C BDE-49 pretreatment (n=294) enhances caffeine-triggered $[Ca^{2+}]_i$ release in RyR1-HEK293 at suboptimal, but not maximal, caffeine concentrations. No significant effects were observed in caffeine responses in HEK293^{RyR1} cells pretreated with BDE-47 (n=158) or vehicle (n=272). Data were collected and analyzed from individual cells on different culture days. *$p<0.05$. D Western blotting with monoclonal antibody C3-33 recognizes RyR2 in membrane homogenates of HEK293 cells stably transfected with RyR2 (HEK293^{RyR2}), but not in preparations of HEK293^{null} cells. E Representative traces of Ca^{2+}-transients elicited by 10 sec focal application of the RyR2 agonist 4-CmC (1mM, bar) to HEK293^{RyR2} cells pretreated with vehicle or 250nM BDE-49 for 16 hr. 4-CmC failed to sensitize Ca^{2+}-transients in HEK293^{null} cells (not shown). F Compared to vehicle control (n=56), pretreatment with BDE-49 (n=69), but not BDE-47 (n=34), for 16 hr significantly enhanced response to 4-CmC. *$p<0.05$